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Reactivity Pattern of a Monoclonal Antikeratin Antibody (KL1)

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A monoclonal antibody against keratins (KL1) from normal human stratum corneum was obtained using hybridoma techniques. Spleen cells from immunized BALB/c mice were fused with NS1, a mouse myeloma cell line, to produce hybrids. Antibody activity to epidermal keratins was tested using an indirect immunofluorescence test on cryostat sections of human epidermis and rabbit lip. A stable clone producing antikeratin antibody was isolated and an ascitic fluid was produced and used as a source of antibody (IgG1 kappa). KL1 was characterized by its immunohistochemical staining of various epithelia and by its recognition of 55–57 kilodalton (kd) keratin polypeptide from normal epidermis using the immunoblot technique. Frozen and deparaffinized sections of normal human epidermis, mucosa, and esophagus were stained by this antibody only in the upper cell layers as demonstrated by both indirect immunofluorescence and immunoperoxidase techniques. Approximatively 80% of normal keratinocytes isolated after trypsinization were labeled by KL1 whereas most negative cells showed basement membrane zone antigens. This confirmed differences in the expression of medium-sized polypeptides between basal and suprabasal cells during the course of human epidermal differentiation. All epithelial cells from other human epithelia (thymus, thyroid, bronchial mucosa, stomach, intestines) were positive with KL1 whereas nonepithelial cells and tissues did not show any staining. In view of these results KL1 promises to be a useful tool in the exploration of human epithelial differentiation.

Epithelial cells (EC) are characterized by a cytoskeleton of keratins which can be immunologically stained using antikeratin antibodies [1–5]. However, these EC have a morphology and specialization which differ according to their species of origin, and whether they belong to stratified squamous epithelia of ectodermal and endodermal origin or to epithelia other than stratified squamous. Cells from different epithelia do not contain identical keratins even though most of them may be stained by polyclonal antikeratin antisera. The origin of this heterogeneity is in the number and size of multiple keratin polypeptide components, which vary with the species and tissue of origin [6–9]. Keratin polypeptides are the products of different genes and are expressed in cells at different stages in their differentiation process [10–12]. In the human epidermis, subsets of keratinocytes can be recognized using polyclonal antibodies against keratin polypeptide bands of different M_r [13,14] or by monoclonal antibodies (MCA) [15,16]. These MCA are of great use in providing information on complex antigens such as keratins by permitting the analysis of one antigenic determinant at a time.

In the present study we describe the properties of a MCA against keratins from normal human stratum corneum and its reactivity pattern on various human tissues.

MATERIALS AND METHODS

Keratins

Samples of stratum corneum (SC) were obtained from the heels of human volunteers who had no skin or internal disorders. Healthy skin was obtained from 10 different patients undergoing plastic surgery (8 samples of breast skin and 2 samples of abdominal wall skin). Whole epidermis was prepared from the skin by trypsinization according to a technique already described [17].

Keratins were purified from the above samples according to the technique described by Winter et al [18]. The pellet obtained after several homogenizations of the SC or epidermis in high-salt buffer was dissolved in 10 mM Tris-HCl pH 8.0, 0.5% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol, and heated for 2 h at 60°C.

The purification and analysis of keratins were controlled by polyacrylamide gel electrophoresis (PAGE) in an 8% slab gel using SDS as already described [14].

Preparation of monoclonal antibody

BALB/c mice were immunized by injecting 50 μg of purified sc keratin emulsified in complete Freund's adjuvant i.p. into the foot pads. They were boosted 14 days later in the same manner. Eight days later the presence of antikeratin antibodies was tested by immunofluorescence (IF) on skin sections. The sera tested were consistently positive (at dilution 1:64). The mice were then ready for fusion, which was performed 3 days after a booster injection of 50 μg of keratin i.v. without adjuvant. Splenocytes (20×10^6) from an immunized mouse were fused with 4×10^6 NS1 myeloma cells according to the technique of Kohler and Milstein [19]. From 3 different fusions, 233 colonies were found 13 days later. The supernatants of these were screened by IF on skin sections. Ten supernatants were found to be positive and the 10 corresponding colonies were cloned. Two lines secreting antibodies were obtained from 2 different colonies, KL1 and KL2. KL2 appeared further to secrete an antibody against nuclear elements and was not used in this study, whereas KL1 was characterized as an antikeratin antibody (see *Results*). By immunodiffusion KL1 was found to belong to the IgG1 class. This line was further subcloned to confirm its monoclonality. KL1 antibodies were produced either in culture or by induction of ascites in BALB/c mice. Ammonium sulfate (50% saturation) precipitates of culture supernatants or of ascites were dialyzed against phosphate-buffered saline (PBS), aliquoted, and stored at −70°C. Once defrozen the aliquots could be kept for several weeks at 4°C with 0.02% sodium azide as a preservative.

Indirect IF Tests

Frozen sections (4 μm) of normal human epidermis (foreskin) and rabbit lip were fixed in acetone (−20°C), rinsed in PBS, and incubated with either the supernatant of the culture fluids or the ascitic fluids for 30 min. After washing in PBS (30 min) sections were incubated with a

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Abbreviations:
BSA: bovine serum albumin
EC: epithelial cells
FITC: fluorescein isothiocyanate
IF: immunofluorescence
kd: kilodaltons
MCA: monoclonal antibody
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
SC: stratum corneum
SDS: sodium dodecyl sulfate

1:20 diluted goat antimouse fluorescein isothiocyanate (FITC) conjugate (Meloy) for 30 min. After another washing in PBS, the slides were mounted with polyvinyl alcohol medium [20]. Control reactions included frozen sections (1) treated with the conjugate alone; (2) incubated with either culture medium or another unrelated ascitic fluid without antikeratin activity.

Immunologic Reactivity of KL1 with Keratin Polypeptides "Blotted" on Nitrocellulose Paper

Keratin polypeptides separated by gel electrophoresis were transferred to nitrocellulose sheets according to the technique described by Towbin et al [21]. The electrophoretic blots were soaked in 5% bovine serum albumin (BSA) in saline (0.9% NaCl, 10 mM Tris-HCl pH 7.4) for 1 h at 40°C and 24 h at 4°C. They were incubated either with KL1 MCA diluted in 3% BSA in saline (1:2000) or with conventional antikeratin antibodies from rabbits immunized with whole human purified keratin [17]. The sheets were washed with 6 changes of buffer and incubated for 2 h at room temperature with fluorescein-conjugated goat antimouse IgG or goat antirabbit IgG (Nordic) (1:30 dilution in saline containing 3% BSA).

Reactivity Pattern of KL1

The reactivity of KL1 was tested by IF or using immunoperoxidase procedures [17] on both frozen sections and sections of paraffin-embedded tissues which were deparaffinized with xylol and hydrated through an alcohol series. Human tissues of diverse origins were obtained from biopsy material fixed in Bouin's solution (Clinique Dermatologique et Service d'Anatomopathologie).

Normal human tissues (epidermis, mucosa, thymus, bronchial mucosa, intestines) were tested with KL1 at 10^{-3} dilution.

Double-Staining Experiments on Cell Smears with FITC and Tetramethyl Rhodamine Isothiocyanate (TRITC) Conjugates

EC were obtained from fresh surgical specimens of normal human skin incubated in 0.25% trypsin (1:250 Difco, Chicago) at 4°C for 12–15 h. Drops of epidermal cell suspensions at a concentration of 10^6 cells/ml were placed on glass slides, air dried, and fixed with acetone. The slides were first incubated wtih KL1, washed, then incubated with goat antimouse IgG-FITC; after further washings, they were incubated with human serum containing antibodies to basement membrane zone antigen at 1:20 dilution, and finally with sheep antihuman IgG TRITC (Kirkegaard-Perry Laboratories, molar f/p ratio 6.8, working dilution 1:20). Normal human and mice sera were used as controls.

Slides were viewed with a Zeiss fluorescence microscope using selective excitation filters for fluorescein and rhodamine. Photographs were taken on 160 ASA Ektachrome films.

RESULTS

The purified keratin proteins were a very effective immunogen since a good antibody response from all BALB/c mice was observed by IF (titer 64) in the samples obtained before the booster immunization preceding cell fusion. From 10 colonies having shown a staining on skin sections, only 2 gave rise to the selection of a stable monoclonal line and only 1 (KL1) was found to produce antikeratin antibodies. By immunodiffusion analysis KL1 was found to be an IgG1 with kappa light chains.

Immunologic Reactivity of KL1 with Keratin Polypeptides

The keratin proteins purified from normal SC and normal epidermis were subjected to electrophoresis in SDS-PAGE and were always compared on a single slab gel. After staining with Coomassie Blue, differences were mainly restricted to the minor bands [<55 kilodalton (kd)]. There was slight variability in the relative quantities of the small polypeptides among individuals, but the number of major bands was similar.

To identify the antigen target of KL1, keratins were transferred to nitrocellulose sheets, where we obtained a replica of all bands detected in slab gels. In the keratins purified from normal human sc (i.e., the antigen used for the mice immunization), KL1 recognized a determinant present in 2 polypeptide groups of 55–57 kd and 63–67 kd. In the keratins purified from human epidermis, KL1 reacted only with the polypeptide group of 55–57 kd. In contrast to results with keratins from SC, no reaction band was detected with higher M_r polypeptides (Fig 1). This KL1 reactivity with a single band group of medium-sized M_r was systematically observed in 8 different cases of breast epidermis and 2 cases of abdominal wall epidermis.

IF Reactivity Pattern (Fig 2)

It was noted on frozen or deparaffinized sections of normal human epidermis, mucosa, and esophagus that KL1 labeled EC from the upper layers whereas those of the basal layer remained negative (Fig 2*A*, *B*). In the dermis, hair follicles, sweat, and sebaceous gland ducts showed similar strong fluorescence with the basal layer remaining negative.

On keratinocyte smears, KL1 strongly labeled about 80% of the cells (Fig 2*C*). In the double-staining experiments, most of the cells negative to KL1 reacted with a human serum containing anti-basement membrane antibodies and demonstrated a line of staining confined to one side of the cells (Fig 2*C1*, *C2*). Some cells were negative to both KL1 and the basement membrane antiserum. EC of all other tissues tested were strongly labeled by KL1, i.e., epithelial reticular cells and Hassall's corpuscles of thymus (Fig 2*D*), thyroid (Fig 2*E*), bronchial mucosa and surface epithelium of stomach (Fig 2*F*), gastric glands, duodenal mucosa, intestinal villi, surface epithelium of the colon, and intestinal glands (Fig 2*G–H*).

All nonepithelial cells tested in human tissues were negative with KL1.

Similar results were obtained with indirect immunoperoxidase studies on paraffin sections.

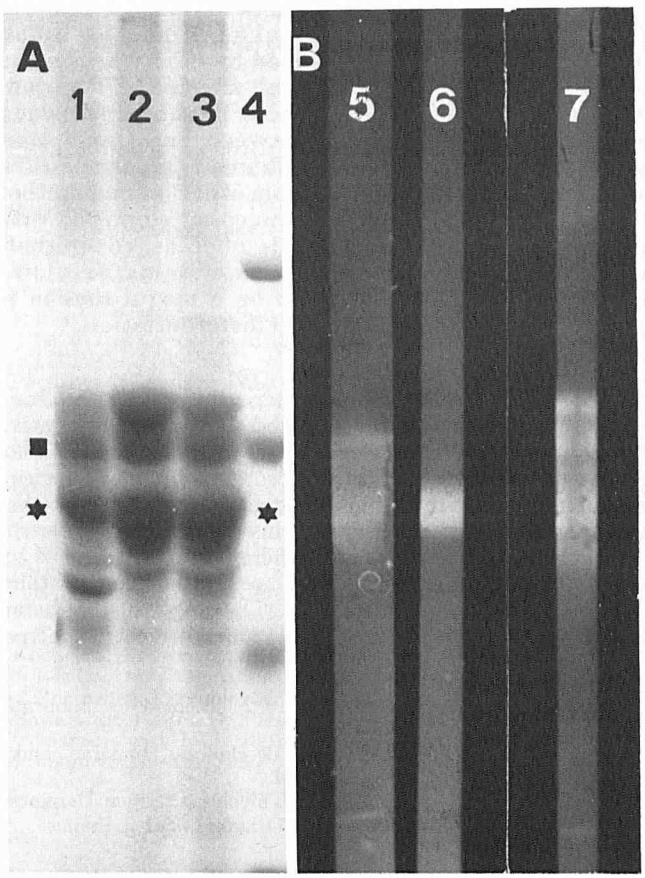
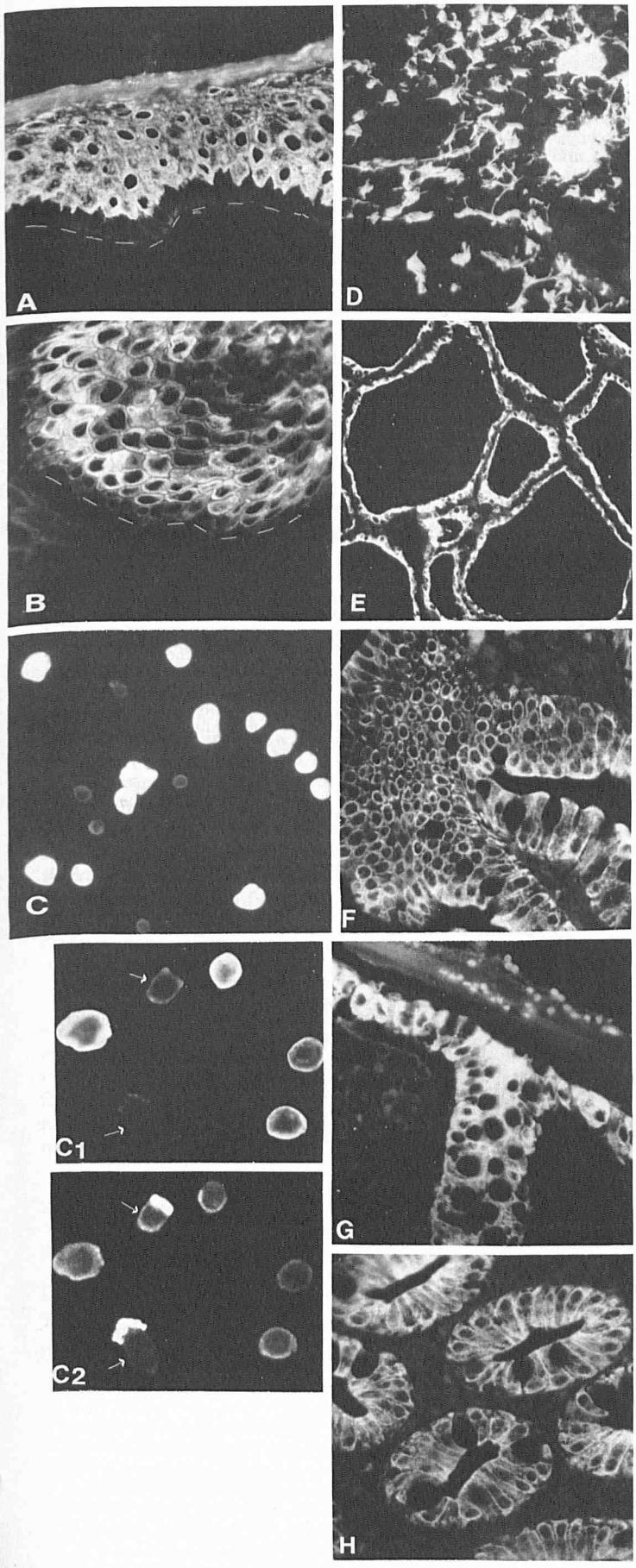


FIG 1. *A*, Analysis by SDS-PAGE of keratins. *Lane 1*, human SC (foot calli); *lanes 2–3*, human epidermis from 2 different breast specimens; *lane 4*, markers. *B*, Immunoblot analysis of keratins. *Lanes 5–6*, human SC and epidermis reacting with KL1 antibody; *lane 7*, human epidermis reacting with a conventional antikeratin antibody. In epidermis only 1 band (55–57 kd) was stained with KL1 whereas 2 bands (55–57 kd and 63–67 kd) reacted in SC. Polyclonal antikeratin antibodies reacted with the main polypeptide bands (49–70 kd).



DISCUSSION

The present report describes the properties of a MCA against keratins that reacts with epidermal upper-layer cells. A particular characteristic of MCA is that they can provide precise information on the expression of a single antigenic determinant in a group of complex cellular antigens. This is of special importance in the case of keratins which are products of a large gene family and whose heterogeneity is reflected in the distinct protein subunits expressed in different cell types including cells from different regions of the same epithelium [5–23]. At least 19 different human cytokeratin polypeptides have been distinguished to date [24]. The immunoblot technique allows an identification of the immunogenic keratin component recognized by KL1. This identification was performed with the keratins from SC and with the cytokeratins from breast and abdominal epidermis. Among the keratins purified from epidermis, only 1 group of bands reacted with KL1 corresponding to the 55–57 kd polypeptides. However, using keratins purified from heel SC, 2 band groups corresponding to the 55–57 kd and the 63–67 kd polypeptides reacted with KL1.

This result shows that 2 polypeptide groups of SC keratins, high-sized (63–67 kd) and medium-sized (55–57 kd), contain a common epitope. Such a result has also been reported for other antikeratin MCA [16–22]. The lack of reactivity of KL1 in immunoblotting with the 63–67 kd polypeptide group present in epidermis is more surprising and indicates subtle immunologic differences between the 2 molecule groups from SC and epidermis. Even if these polypeptides are products of the same genes in both tissues, posttranslational changes occurring during the last steps in the keratinization process could result in the observed immunologic heterogeneity. Such modifications could include, for example, enzymatic hydrolysis, disulphide bond stabilization, and phosphorylation known to occur in the assembly of the fibrous amorphous matrix of the mature corneocytes. Alternatively, sequence heterogeneity may exist within a specific class of intermediate filament proteins, even among those of similar M_r [25].

Whether these differences are intrinsic features of keratinization or are also related to the particular anatomic location of epidermis cannot be determined at the present time. More precise investigations using two-dimensional gel electrophoresis and immunoblotting analysis will be required to answer such questions.

The IF reactivity of KL1 on frozen or deparaffinized tissue sections allowed comparisons among different human epithelia. In epidermis (and stratified squamous epithelia) KL1 reactivity confirmed that high M_r polypeptides present in SC and a group of medium-sized keratins from epidermis are not detected in basal layer cells, as previously shown by the use of polyclonal antibodies directed against either high M_r or medium-sized keratin polypeptides from SC [13,14] and also more recently with MCA [15,16]. However, the analysis of keratin polypep-

FIG 2. Indirect IF staining patterns of human tissues by KL1 antibody on frozen or deparaffinized sections. *A and B*, Epidermis and esophagus with negative basal layer cells (× 500). *C*, Human keratinocytes isolated after trypsinazation. A few negative cells were present in the suspension (× 400). *C1 and C2*, Double-staining experiment on isolated keratinocytes. *C1*, Labeling of keratinocytes with KL1 visualized by fluorescein conjugate. Two cells are negative (*arrows*). *C2*, The same cells, viewed through filters that transmit rhodamine fluorescence, indicating labeling by human serum containing anti-basement membrane zone antigens. The 2 cells negative for KL1 possess these antigens typical of basal layer cells (*arrows*) (× 630). *D*, Epithelial cells and Hassall's corpuscles of thymus (× 250). *E*, Thyroid (× 250). *F*, Surface epithelium of stomach (× 500). *G and H*, Surface epithelium of colon and intestinal glands (× 500).

tides in KL1-negative basal cells remains to be done to determine the nature and the reactivities of their polypeptides; particular keratin polypeptides may be present in these cells, but in an immunologically unrecognizable form. To resolve this problem, analysis of keratin polypeptides in defined cell populations is currently being investigated in cells separated by a fluorescence-activated cell sorter. Recent work by Moll et al [24] on the expression of human cytokeratins in normal epithelia have shown that keratin components of 58 kd and 56 kd are closely related to each other as judged from peptide maps and are observed in various proportions in many nonkeratinizing stratified squamous epithelia of humans, in epidermis and hair follicles. It is not unlikely that KL1 reacts specifically to this keratin group, more precisely with the 56 kd keratin, the 58 kd keratin being present in the basal layer as recently reported [26]. It may also recognize some other keratins in these epithelia, thus explaining the variable IF staining patterns. However this hypothesis remains to be confirmed by immunoblotting analysis.

In conclusion, the IF reactivity pattern of KL1 indicates that it can be considered as a good marker of human cytokeratins since all EC tested were strongly positive with the exception of basal cells of stratified squamous epithelia. Nonepithelial cells and tissues were all negative when reacted with KL1.

In view of these results, and the subtle differences observed in its reactions on immunoblots of SC and epidermal keratins, KL1 promises to be a useful tool in the exploration of human epithelial differentiation.

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